Low pH and an Intracellular Environ Induce *Staphylococcus epidermidis* Small Colony Variant Formation

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Abstract

Background: Small colony variants (SCVs) are naturally occurring, slow-growing subpopulations of bacteria that emerge in response to diverse environmental pressures and that may persist intracellularly. The slow growth of SCVs may compromise antimicrobial activity. In addition, their intracellular survival may shield them from the host immune system and from extracellularly active antimicrobials. Moreover, SCVs may be undetected by conventional diagnostic tests. This may result in the discontinuation of antimicrobial treatment before SCVs are effectively cleared, resulting in their persistence and association with recurrent infection. It is unclear how SCVs are induced and maintained in patients. We assessed the effect of low pH and intracellular growth on the selection of S. epidermidis SCVs in vitro.

Methods: S. aureus strain 6850 (known to form SCVs under low pH conditions), S. epidermidis strain RP62A and two S. epidermidis prosthetic joint infection isolates, IDRL-8933 and IDRL-8864, were studied. Bacteria were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) at 37˚ C in 5% CO2 for 5 days with quantitative cultures performed on days 0, 3, and 5. Adjusted pH values of 4, 5.5, and 6.5 were achieved mixing the EMEM 10% FBS medium with buffers containing Na2HPO4 and citric acid of pH 2.6, 4.0, and 5.6. pH 7.4 was maintained by addition of 50 mM HEPES. In addition, human lung fibroblast MRC5 cells were infected at a multiplicity of infection of 1 for intracellular assay. MRC5 cells were washed daily with PBS and lysostaphin (to kill extracellular bacteria). At 0, 3, 5 and 7 days post infection, host cells were lysed and serial 10-fold dilutions of the cell lysates plated onto sheep blood agar plates to detect and quantitate intracellular bacteria. Colony-forming units (CFUs) were enumerated after overnight incubation at 37˚ C and the colony phenotype determined after an additional incubation overnight at room temperature. SCVs were identified on the basis of their size, reduced pigmentation and reduced hemolysis.

Results: The frequency of SCVs was increased over time as the pH in the medium was decreased for all four strains tested. Additionally, the frequency of SCVs increased in the intracellular environment of human lung fibroblast cells. At day 0 after 3 hours of infection, the frequency of SCVs was below 7% for each strain. By day 7, on average, 23% of the viable intracellular bacteria were SCVs.

Conclusion: Low pH and an intracellular environ promote the formation of SCVs. Acidic environments, such as within lysosomes or phagosomes, may induce S. epidermidis SCV-formation.

Background

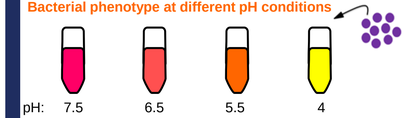
Small colony variants (SCVs) are naturally occurring, slow-growing subpopulations of bacteria that emerge in response to diverse environmental pressures and that may persist intracellularly. They typically comprise a minor proportion of the source population and are common in chronic infections. Particularly, *Staphylococcus epidermidis* SCVs are common in prosthetic joint infection (PJI).

Objectives

Assess the effect of low pH and intracellular growth on the selection of *S. epidermidis* SCVs in vitro

Methods

Bacterial phenotype at different pH conditions



Infect media with bacteria
Incubate, shaking at 37°C in 5% CO_2 for up to 5 days
pH and OD_{600} measured daily
At respective time points: D0, D3, and D5, plate serial dilutions

Bacterial phenotype during intracellular persistence

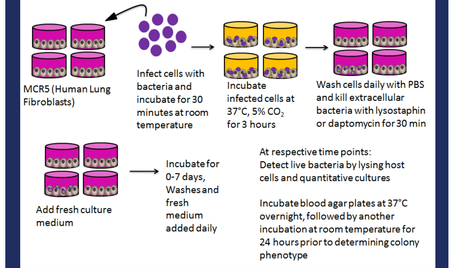


Figure 1: Low pH induces SCV formation

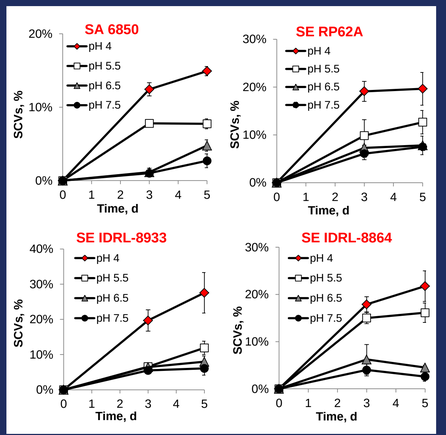
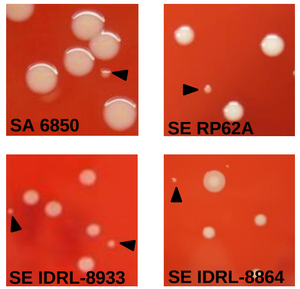


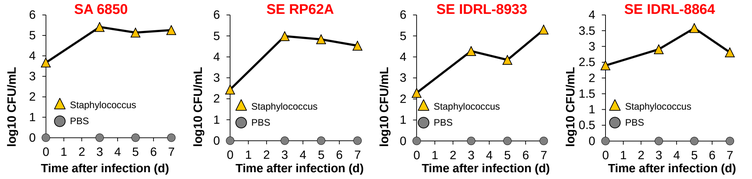
Figure 2: Colony phenotype



Shown are representative images of each isolate, grown on sheep blood agar plates, containing both normal phenotypes and SCVs. Colony phenotype was assessed by colony size and hemolysis. **Black arrows denote SCVs.**

Figure 3: Frequency of SCVs overtime during intracellular infection

The number of Viable Colony Forming Units (CFU) per mL Overtime



The Percentage of Intracellular Persisting SCVs Overtime

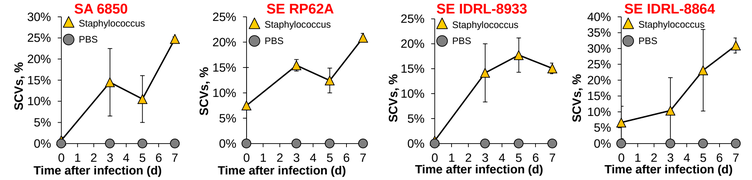
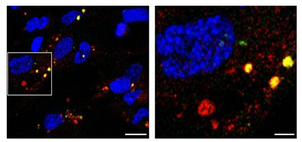


Figure 4: Localization of Intracellular *S. epidermidis*



Internal localization of persisting bacteria (FITC; green) was analyzed by fluorescence microscopy three hours after infection. Lysosomes were visualized with LAMP-2 antibody (Alexa Fluor 594; red) and nuclei were stained with DAPI (blue). Scale bars 20 µm and 5 µm, respectively.

Results

Figure 1 and 2: 30 minutes after inoculation, each strain showed a normal colony phenotype, independent of pH. The frequency of SCVs increased over time at pH 4.0, ultimately reaching >15%, while at pH 7.4, SCVs were noted at a frequency of <8%. At pH 5.5 and 6.5, there was an intermediate percentage of SCVs. These results show a positive correlation between low pH and SCV formation in three *S. epidermidis* strains.

Figure 3: The number of viable intracellularly persisting bacteria remained fairly constant over the course of infection, while the frequency of SCVs increased in the intracellular environment, reaching an average of 23% after 7days from an average 4% after three hours. These results show a positive correlation between intracellular persistence and SCV formation in three *S. epidermidis* strains.

Figure 4: Our data suggested that low pH favors *S. epidermidis* SCV formation, and that the intracellular environment may have an analogous effect. Intracellular bacteria were found to be localized within LAMP-2 antibody-positive vesicles, suggesting that intracellularly persisting *S. epidermidis* primarily reside within phagolysosomes. Samples stained with IgG isotype control had negative results (not shown).

Conclusions and Discussion

- Low pH and an intracellular environ promote the formation of SCVs. Acidic environments, such as within lysosomes or phagosomes, may induce *S. epidermidis* SCV-formation.
- It has also been shown that *S. epidermidis* SCVs may revert back to fast growth, *in vitro*. Although unknown *in vivo*, their intracellular persistence and SCV formation may be a strategy *S. epidermidis* uses to cause chronic PJI. Localization within host cells may shield *S. epidermidis* from antibiotics will poor cell penetration, antimicrobials, and immune cells.
- SCV formation has been shown to be triggered by antibiotic pressures and different environmental stresses. These observations along with our findings emphasize how numerous stimuli may induce or promote *S. epidermidis* SCV formation.

Future Directions

- Elucidate whether acidic pH selects for or induces SCVs.
- Determine whether neutralizing pH in lysosomes would decrease the frequency of SCVs.
- Determine if *S. epidermidis* escapes the phagolysosome.
- Examine intracellular persistence of *S. epidermidis* in human osteoblasts, macrophages, and/or neutrophils

References

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